Exhibit B

Guggenheim, S., & Flavin, M. (1969b) *J. Biol. Chem. 244*, 6217-6227.
Guggenheim, S., & Flavin, M. (1971) *J. Biol. Chem. 246*, 3562-3568.
Holbrook, E. (1984) Doctoral Dissertation, Duke University.
Holbrook, E. L., Greene, R. C., & Krueger, J. H. (1990) *Biochemistry* (preceding paper in this issue).
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 133-146, McGraw-Hill, New York.
Johnston, M., Marcotte, P., Donovan, J., & Walsh, C. (1979a) *Biochemistry 18*, 1729-1738.
Johnston, M., Jankowski, D., Marcotte, P., Tanaka, H., Esaki, N., Soda, K., & Walsh, C. (1979b) *Biochemistry 18*, 4690-4701.
Johnston, M., Raines, R., Chang, M., Esaki, N., Soda, K., & Walsh, C. (1981) *Biochemistry 20*, 4325-4333.
Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., Metzler, D. E., Morozov, Y. V., Ralston, I. M., Savin, F. A., Torchinsky, Y. M., & Ueno, H. (1985) *The Transaminases*, pp 37-105, Wiley, New York.
Kaplan, M. M., & Flavin, M. (1966) *J. Biol. Chem. 241*, 4463-447.
Karube, Y., & Matsushima, Y. (1977) *J. Am. Chem. Soc. 9*, 1356-1358.
Koerber, S. C., MacGibbon, A. K. H., Dietrich, H., Zeppequaer, M., & Dunn, M. F. (1983) *Biochemistry 22*, 3424-3431.
Metzler, D. E., Harris, C. M., Johnson, R. J., Siano, D. B., & Thomson, J. A. (1973) *Biochemistry 12*, 5377-5392.
Posner, B. I., & Flavin, M. (1972a) *J. Biol. Chem. 247*, 6402-6411.
Posner, B. I., & Flavin, M. (1972b) *J. Biol. Chem. 247*, 6412-6419.
Tanizawa, K., & Soda, K. (1979) *J. Biochem. 86*, 1199-1209.
Tate, S. S., & Meister, A. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 35*, 503-543.
Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, pp 777-827, Freeman, San Francisco.
Washtien, W., Cooper, A. J. L., & Abeles, R. H. (1977) *Biochemistry 16*, 460-463.

Phase Equilibria of Cholesterol/Dipalmitoylphosphatidylcholine Mixtures: ^2H Nuclear Magnetic Resonance and Differential Scanning Calorimetry†

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ABSTRACT: Deuterium nuclear magnetic resonance spectroscopy and differential scanning calorimetry are used to map the phase boundaries of mixtures of cholesterol and chain-perdeuteriated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine at concentrations from 0 to 25 mol % cholesterol. Three distinct phases can be identified: the L_α or liquid-crystalline phase, the gel phase, and a high cholesterol concentration phase, which we call the β phase. The liquid-crystalline phase is characterized by highly flexible phospholipid chains with rapid axially symmetric reorientation; the gel phase has much more rigid lipid chains, and the motions are no longer axially symmetric on the ^2H NMR time scale; the β phase is characterized by highly ordered (rigid) chains and rapid axially symmetric reorientation. In addition, we identify three regions of two-phase coexistence. The first of these is a narrow L_α/gel-phase coexistence region lying between 0 and about 6 mol % cholesterol at temperatures just below the chain-melting transition of the pure phospholipid/water dispersions, at 37.75 °C. The dramatic changes in the ^2H NMR line shape which occur on passing through the phase transition are used to map out the boundaries of this narrow two-phase region. The boundaries of the second two-phase region are determined by ^2H NMR difference spectroscopy, one boundary lying near 7.5 mol % cholesterol and running from 37 down to at least 30 °C; the other boundary lies near 22 mol % cholesterol and covers the same temperature range. Within this region, the gel and β phases coexist. As the temperature is lowered below about 30 °C, the phospholipid motions reach the intermediate time scale regime of ^2H NMR so that spectral subtractions become difficult and unreliable. The third two-phase region lies above 37 °C, beginning at a eutectic point somewhere between 7.5 and 10 mol % cholesterol and ending at about 20 mol %. In this region, the L_α and β phases are in equilibrium. The boundaries for this region are inferred from differential scanning calorimetry traces, for the boundary between the L_α- and the two-phase region, and from a dramatic sharpening of the NMR peaks on crossing the boundary between the two-phase region and the β-phase region. In this region, the technique of difference spectroscopy fails, presumably because the diffusion rate in both the L_α- and β-phase domains is so rapid that phospholipid molecules exchange rapidly between domains on the experimental time scale.

Cholesterol is a major constituent of the plasma membrane of many of the cells of higher organisms, making up as much as 50 wt % of the lipid fraction in the case of the human erythrocyte membrane. Even so, its functional role within the membrane is not understood. The large changes which it induces in the physical properties of membranes suggest that part of its function may be to improve these characteristics over those of, for example, a simple phospholipid bilayer. Additionally, it may permit wider variations in composition of the other membrane constituents while maintaining the

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integrity of the membrane under varying physiological conditions. In order to investigate these possibilities, it is first necessary to quantitate the changes in these physical properties. The first step is to study the phase equilibria of a simple two-component phospholipid/cholesterol system.

The effect of cholesterol on the physical properties of phospholipid bilayers has been studied extensively. Mixtures of cholesterol with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)[1] have been studied by electron paramagnetic resonance (EPR) (Oldfield & Chapman, 1971; Schreier-Mucillo et al., 1973; Shimshick & McConnell, 1973; Delmelle et al., 1980; Presti & Chan, 1982; Kar et al., 1985), nuclear magnetic resonance (NMR) (Gally et al., 1976; Haberkorn et al., 1977; Brown & Seelig, 1978; Kuo & Wade, 1979; Lindblom et al., 1981; Jarrel et al., 1981; Wittebort et al., 1982; Vist, 1984; Davis, 1988), fluorescence polarization (Fahey & Webb, 1978; Kawato et al., 1978; Lentz et al., 1980; Wolber & Hudson, 1981; Kutchai et al., 1983), X-ray diffraction (Ladbrooke et al., 1968; McIntosh, 1978; Rand et al., 1980; Alecio et al., 1985, differential scanning calorimetry (DSC) (Ladbrooke et al., 1968; Hinz & Sturtevant, 1972; Mabrey et al., 1978; Gershfeld, 1978; Estep et al., 1978; Vist, 1984; Gentz et al., 1986; Offringa et al., 1987), ac calorimetry (Imaizumi & Hatta, 1984), freeze-fracture (Copeland & McConnell, 1980; Lentz et al., 1980), dilatometry (Melchior et al., 1980), viscoelastic measurements (El-Sayed et al., 1986), and dielectric relaxation (Shepherd & Buldt, 1979; Henze, 1980).

A similar system, mixtures of cholesterol with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), has been equally widely studied and found to behave similarly using the above techniques: EPR (Shimshick & McConnell, 1973; Rubenstein & McConnell, 1980; Recktenwald & McConnell, 1981; Presti & Chan, 1982), NMR (Oldfield et al., 1971, 1978; Haberkorn et al., 1977; Jacobs & Oldfield, 1979; Cornell et al., 1982; Westerman et al., 1982; Wittebort et al., 1982; Cornell & Keniry, 1983), fluorescence polarization (Alecio et al., 1982; Smutzer & Yeagle, 1985; Deinum et al., 1988; Smutzer, 1988), X-ray diffraction (Schwartz & Paltauf, 1976; McIntosh, 1978; Hui & He, 1983; Alecio et al., 1985), DSC (Hinz & Sturtevant, 1972; Mabrey et al., 1978; Calhoun & Shipley, 1979), freeze-fracture (Copeland & McConnell, 1980), dielectric relaxation (Henze, 1980), neutron scattering (Knoll et al., 1985; Mortensen et al., 1988), and micromechanical measurements (Needham et al., 1988).

In addition, there have been many studies of the effect of cholesterol on bilayers using other phosphatidylcholine (PC) lipids. Some of these which provide useful comparisons with or are supplementary to DPPC/cholesterol studies are the following: EPR (Oldfield & Chapman, 1971; Schreier-Mucillo et al., 1973; Taylor & Smith, 1980; Dahl, 1981; Presti & Chan, 1982); NMR (Stockton et al., 1974, 1976; Opella et al., 1976; Stockton & Smith, 1976; deKruijff, 1978; Lindblom et al., 1981; Brainard & Cordes, 1981; Cornell et al., 1982; Tilcock et al., 1982); fluorescence polarization (Hildebrand & McConnell, 1979; Kutchai et al., 1983; Vincent & Gallay, 1984; van Ginkel et al., 1986; Deinum et al., 1988); X-ray diffraction (Franks, 1976; McIntosh, 1978); neutron diffraction (Worchester & Franks, 1976).

Nuclear magnetic resonance studies of mixtures of cholesterol with phosphatidylethanolamines (PE's) have found effects simlar to those found with PC's (Brown & Seelig, 1978; Blume & Griffin, 1982; Wittebort et al., 1982; Ghosh & Seelig, 1982; Jarrell et al., 1987) as have NMR studies of the membranes of *Acholeplasma laidlawii* grown on deuteriated fatty acids and cholesterol (deKruijff et al., 1976; Davis et al., 1980; Rance et al., 1982) and of human erythrocyte ghosts (Davis et al., 1979a; Maraviglia et al., 1982). There have been a large number of review articles which were concerned at least in part with the effect of cholesterol on PC bilayers (Phillips, 1972; Chapman, 1975; Demel & deKruijff, 1976; Lee, 1977; McElhaney, 1982; Davis, 1983; Cullis et al., 1985; Yeagle, 1985).

Some of the principal effects which have been observed in the above studies are as follows: (i) a dramatic increase in orientational order of the phospholipid hydrocarbon chains, or of EPR or fluorescent probe molecules; (ii) the concomitant thickening of the hydrophobic part of the bilayer; (iii) the broadening and eventual elimination of the "phase transition" from the higher temperature liquid-crystalline phase to the lower temperature gel phase; (iv) suppression of the pretransition; and (v) a decrease in the chain tilt angle in the gel phase. A few of the papers cited above interpret their results in terms of partial phase diagrams (Ladbrooke et al., 1968; Shimshick & McConnell, 1973; Gershfeld, 1978; Lentz et al., 1980; Rand et al., 1980; Recktenwald & McConnell, 1981; Blume & Griffin, 1982; Mortensen et al., 1988). Each of these points will be discussed below in light of a new interpretation of the phase equilibria of the cholesterol/DPPC system.

There have been a number of theoretical efforts at understanding the origin of the cholesterol-induced changes in the bilayer (Marcelja, 1974; Pink & Carrol, 1978; Scott & Cheng, 1978; Cornell et al., 1979; Pink & Chapman, 1979; Owicki & McConnell, 1980; Jahnig, 1981a,b; Gruen, 1982; Mouritsen et al., 1983), and very recently there has been a theoretical description of the phase behavior of DPPC/cholesterol mixtures (Ipsen et al., 1987) based on a preliminary report (Vist, 1984) of the results presented here.

We examine the phase equilibria of these mixtures using the techniques of deuterium NMR and differential scanning calorimetry. ^{2}H NMR of chain-labeled DPPC measures directly the degree of orientational averaging, allowing us to quantitate the ordering effect of cholesterol. In addition, the ^{2}H spectrum is sensitive to the symmetry and rates of the motions which allows us to study the coexistence of, for example, liquid-crystalline and gel phases. This approach and its extension in the form of ^{2}H NMR difference spectroscopy, previously applied to a number of other two-component membrane systems (Bienvenue et al., 1980; Huschilt et al., 1985; Morrow et al., 1985, 1986; Morrow & Davis, 1988), will be used in conjunction with DSC to map out the phase boundaries of cholesterol/DPPC between 0 and 25 mol % cholesterol, over the temperature range of from about 25 to 60 °C. The nature of the stable phases and of the two-phase regions which we observe will be discussed in light of the present work and those which preceded it.

The next section describes our experimental approach and discusses the means by which we establish sample homogeneity over the concentration range studied. The major results of the study are presented and analyzed in the succeeding section, and the technique of ^{2}H NMR difference spectroscopy is described. The partial phase diagram is constructed from these results. The concluding section describes the molecular orientational order and dynamics in each of the regions of the phase diagram, bringing together the results of this ^{2}H NMR

[1] Abbreviatons: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; DPPC-d_{62}, 1,2-bis(perdeuteriopalmitoyl)-*sn*-glycero-3-phosphocholine.

and DSC study and those of a large number of earlier experiments.

Materials and Methods

Five grams of cholesterol obtained from Sigma (lot 41F-7415, Sigma grade 99+%; Sigma, St. Louis MO) was dissolved in hot redistilled ethanol and hot-filtered with Whatman W-2 filter paper. The hot solution was allowed to cool, and crystals were observed within 3 h. Once the crystals had begun forming, the flask was placed into a refrigerator for about 1 h. The crystals were then filtered and washed with chilled redistilled ethanol, and finally were dried at room temperature for 1 day under vacuum.

Thin-layer chromatography (TLC) was performed to check the purity of the recrystallized cholesterol. The eluting solvent was ethyl acetate/hexane/acetic acid, 15:4:1 v/v. TLC's were performed on recrystallized cholesterol (both before and after recrystallization), dihydrocholesterol, and cholestane. To aid in viewing the developed plates, they were srayed with sulfuric acid and heated on a hot plate. By TLC, the recrystallized cholesterol gave a single spot readily distinguishable from cholestane and dihydrocholesterol by both color and position. A stock solution of 2.0 mM cholesterol in methanol was prepared from the recrysallized cholesterol.

The phospholipid 1,2-bis(perdeuteriopalmitoyl)-*sn*-glycerol-3-phosphocholine (DPPC-d_{62}) was synthesized according to the procedure described by Gupta et al. (1977) and by Huschilt et al. (1985). A stock solution of 10 mM DPPC-d_{62} in methanol was prepared. As discussed below the ^{2}H NMR quadrupolar splittings are found to be very sensitive to cholesterol concentration in the liquid-crystalline phase. Therefore, if a sample has a heterogeneous distribution of cholesterol concentrations due to poor mixing, the NMR spectrum will exhibit a broad distribution of splittings. In addition, there is a region just above the phospholipid chain-melting transition, at moderate cholesterol concentrations, where the peaks in the spectrum broaden. In this concentration range, we must look at higher temperatures to find the sharp peaks characteristic of a homogeneous sample. Cholesterol/DPPC samples prepared from chloroform solution were found to be heterogeneous, so we tested various solvents and solvent mixtures. Using methanol as a mixing solvent provided us with homogeneous samples throughout the composition range of from 0 to 25 mol % cholesterol.

All of the NMR and DSC samples were prepared by mixing appropriate volumes of the two stock solutions (DPPC-d_{62} and cholesterol) to obtain the desired concentration of cholesterol in phospholipid. The mixture was dried on a Buchi rotovap and then dried further overnight (greater than 12 h) under vacuum at room temperature. The mixture was scraped from the sides of the drying flask to increase the surface area, and the flask was then placed under vacuum again for a few hours.

NMR samples containing DPPC-d_{62} and cholesterol were prepared at the following mole fractions of cholesterol: 0, 0.0125, 0.025, 0.05, 0.0625, 0.075, 0.10, 0.15, 0.20, 0.225, and 0.25. The samples were prepared so that the final total dry weight was approximately 80 mg (of DPPC-d_{62} + cholesterol). The amount of 50 mM phosphate buffer (pH 7.0) added was $^4/_3$ times the dry weight of each sample, typically 105 μL. The samples were mixed by hand using a small glass rod, in 25 mm long, 8-mm diameter NMR tubes. All of the samples were checked for degradation after completion of the NMR experiments by TLC, eluting with $HCCl_3/CH_3OH/H_2O/NH_4OH$, 55:35:5.6:1.6 v/v. Typically, the ^{2}H NMR experiments took 3–4 days per sample, but no degradation of any of the samples could be detected by TLC.

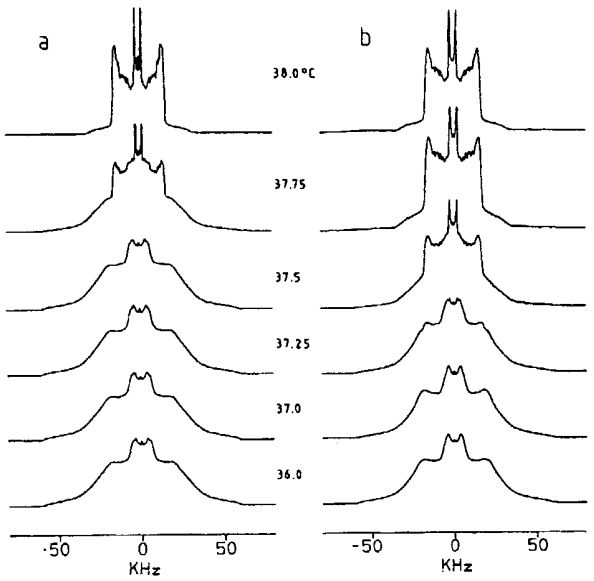

FIGURE 1: ^{2}H NMR spectra at 41.3 MHz of (a) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-d_{62} (DPPC-d_{62}) in excess phosphate buffer near the hydrocarbon chain melting transition and (b) DPPC with 2.5 mol % cholesterol over the same temperature range.

All of the ^{2}H NMR experiments were performed using the quadrupolar echo sequence (Davis et al., 1976) with $\pi/2$ pulse lengths of 2.5 μs. The delay between the two $\pi/2$ pulses in the quadrupolar echo sequence was 35 μs, and the repetition rate was 1.1 s^{-1}. All measurements were made at 41.3 MHz using a home-built spectrometer. All spectra are the result of signal averaging 2000 scans and Fourier transforming from the top of the echo (Davis, 1983). The phases of both pulses used in forming the echo were cycled as described by Davis (1983). Both of the quadrature signals were acquired, and the out of phase component, which for perfectly symmetric spectra should contain only noise, was discarded if it contained no signal. In each case, the echo was symmetrized about its maximum as described by Davis (1983).

Differential scanning calorimetry (DSC) was performed on aliquots taken from the mixtures used for the NMR samples. Usually 2–4 mg of dry lipid was used, and 10–20 μL of buffer was added. This mixture was vortexed and centrifuged in the DSC sample cell. The DSC scans were taken with a Microcal (Microcal Inc., Amherst, MA) MC-1 high-sensitivity differential scanning calorimeter at a scan rate of either 9 or 12 °C/h. The only exception was for the 22.5 mol % sample, where a scan rate of 44 °C/h was used to enhance the strength of the signal. No area, or enthalpy, measurements were attempted on these samples.

Results and Analysis

Experimental Data—NMR and DSC. ^{2}H NMR spectra were obtained at 1 or 2 °C intervals over the temperature range from 0 to greater than 50 °C for samples with concentrations ranging from 0 to 25 mol % cholesterol in DPPC-d_{62}. We were especially interested in the behavior of this system at low cholesterol concentrations, so that 7 of the 11 samples studied had concentrations less than or equal to 10 mol %. Spectra for two samples, pure DPPC-d_{62} and the 2.5 mol % mixture, are shown in Figure 1.

The pure phospholipid undergoes a sharp transition near 37.75 °C (Davis, 1979; Huschilt et al., 1985), as illustrated by the spectra in Figure 1a. At 38 °C, this sample is entirely in its fluid or L_α phase while at 37.5 °C it is in its gel or $P_{\beta'}$ phase (Tardieu et al., 1973; Janiak et al., 1976; Stamatoff et al., 1982; Alecio et al., 1985). At 37.75 °C, two components

are seen in the spectrum, indicating the coexistence of both gel and fluid domains. For a pure single-component system, this is possible only at a single temperature. Insofar as we are careful to ensure that our samples always have an excess of water, i.e., that there exists a bulk water phase, we may treat the DPPC/water system as having effectively a single component. In any real system, the phase transition will have a finite width; for this sample, the width is considerably less than 0.5 °C, as demonstrated by these spectra. The large changes in the width and shape of the ^2H NMR spectrum which occur at phase boundaries will play a fundamental role in our efforts to determine the phase behavior of the cholesterol/DPPC system.

The spectra in Figure 1b are for the sample containing 2.5 mol % cholesterol. Here, at 37.75 °C, the sample is still entirely in its fluid phase, and the coexistence region, with both gel and fluid domains, has broadened to include both 37.5 and 37.25 °C. The transition to the gel phase is not complete until the temperature is lowered to 37 °C. For samples with 1.25 and 5 mol % cholesterol, a similar broadening and slight downward shift of the coexistence region is observed. The spectra of the gel phase immediately below this narrow two-phase region are characteristic of the $P_{\beta'}$ phase. In particular, the central portion of the spectrum, which is due to the chains' methyl groups, has the often observed sloping shoulders indicative of an asymmetric powder pattern (Davis, 1979, 1983; Westerman et al., 1982). The existence of these sloping shoulders has been taken as characteristic of the $P_{\beta'}$ phase. For the pure DPPC-d_{62} sample, the methyl group component of the spectrum broadens, and the sloping shoulders vanish at temperatures below the pretransition from the $P_{\beta'}$ phase to the lower temperature $L_{\beta'}$ phase.

At 6.25 mol % cholesterol, the two-phase coexistence region persists, but the shape of the gel-phase methyl group component has changed and is no longer indicative of the $P_{\beta'}$ phase. At higher cholesterol concentrations, as we shall see below, we see no evidence for a $P_{\beta'}$ phase either in the NMR spectra or in the DSC scans.

From these spectra, it is clear that for chlesterol concentrations below about 6.25 mol % there is a narrow two-phase region, with a maximum width of about 0.75 °C, lying below the chain melting transition at 37.75 °C of the pure deuteriated phospholipid.

DSC traces of samples from 0 to 22.5 mol % cholesterol are shown in Figure 2. The scans for concentrations less than or equal to 6.25 mol % cholesterol are in Figure 2a, those for higher concentrations are in figure 2b. The trace for pure DPPC-d_{62} shows the widely reported features of the pure phospholipid (Morrow et al., 1985, 1988): a subtransition near 14 °C whose enthalpy depends strongly on incubation time at temperatures below 14 °C, a pretransition near 30 °C whose position on the temperature axis is somewhat variable, depending to some extent on the sample's thermal history, and finally, the sharp chain-melting transition occurring at 37.75 °C [about 3.5 °C lower than the corresponding transition of protiated DPPC (Davis, 1979)].

At a cholesterol concentration of 1.25 mol %, the DSC trace is very similar to that of the pure phospholipid. However, at 2.5 mol % cholesterol, there is a a significant broadening and downward shift in the main chain-melting transition. The subtransition and pretransition are still both observed, although in the particular scan displayed in Figure 2a the pretransition for the 2.5 mol % sample is weak; in other scans it shows up more clearly. At 5 mol % cholesterol, there is further broadening of the main transition, and the subtransition and

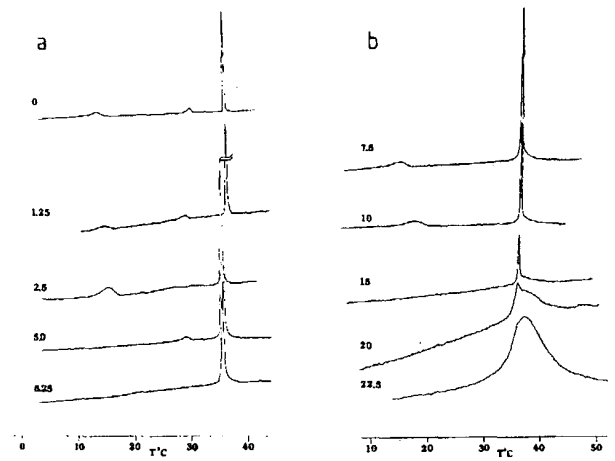

FIGURE 2: Differential scanning calorimetry traces for 10 of the 11 different cholesterol concentrations used in the NMR experiments. Scan rates varied from 9 to 12 °C/h, except for the 22.5 mol % sample where the scan rate was 44 °C/h.

pretransition persist. At 6.25 mol %, the main transition has narrowed again, and the pretransition is not observed in any of the repeated DSC scans.

This calorimetric behavior at low cholesterol concentrations is entirely consistent with our interpretation of the ^2H NMR results on these samples. The major conclusion is that there is a narrow two-phase region between 0 and about 6.25 mol % cholesterol, lying just below the chain-melting transition of the pure phospholipid.

At 7.5 mol % cholesterol, the main transition appears even sharper than at 6.25 mol %, but there is clear evidence of a broad shoulder at the high-temperature side of the sharp peak. As the cholesterol concentration is increased further, the size of the sharp peak decreases, but the temperature at which it occurs remains constant until above about 20 mol % cholesterol where the sharp peak has vanished completely. The broad shoulder increases in relative importance as the concentration is increased until at 22.5 mol % cholesterol only a broad peak remains. The fact that the broad shoulder appears only on the high-temperature side of the sharp peak is significant and provides an important clue in constructing the phase diagram describing this two-component mixture.

These DSC results agree with those reported previously for protiated DPPC/cholesterol mixtures (Ladbrooke et al., 1968; Mabrey et al., 1978). Before attempting to interpret these results in terms of the phase behavior, we need to examine the ^2H NMR spectra at these higher cholesterol concentrations.

^2H NMR spectra of the 15 mol % cholesterol sample are shown in Figure 3. At 60 °C, the individual peaks in the spectrum are very sharp, as they are in the pure phospholipid spectra in the liquid-crystalline phase. The spectra of the 7.5 and 10 mol % samples also have sharply resolved peaks at these higher temperatures. As the temperature is lowered, the quadrupolar splittings for all except perhaps the α-C^2H_2 positions on the *sn*-2 chain increase dramatically. As this occurs, the resonances for positions near the center of the chains merge with those for the positions nearer the headgroup; i.e., more positions contribute to the "plateau" in the quadrupolar splitting profile of the chain, and a larger portion of the chain experiences nearly the same degree of orientational order. Also, close inspection of the spectrum at 45 °C and that at 60 °C reveals that the line widths of the individual peaks are increasing as the temperature is lowered. Furthermore, this increase in line width seems more pronounced for those peaks with larger quadrupolar splittings.

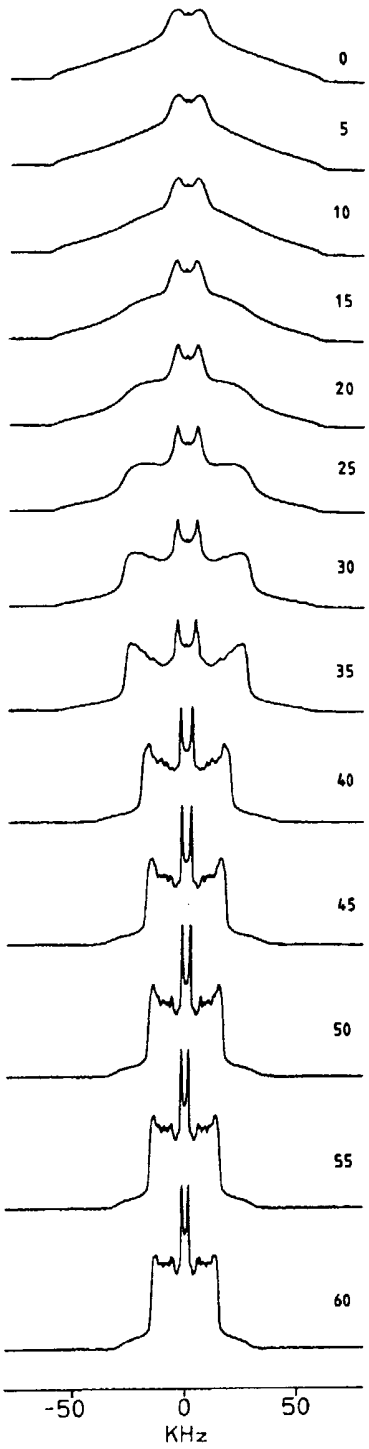

FIGURE 3: ^{2}H NMR spectra of the 15 mol % cholesterol sample as a function of temperature. Temperatures are indicated on the right of each spectrum, in degrees centigrade.

This behavior, at temperatures above 37.75 °C, occurs in all samples with cholesterol concentrations less than 20 mol %. The higher the cholesterol concentration is, the higher the temperature at which these effects become apparent. Figure 4, which displays the ^{2}H NMR spectra at 38 °C for all the samples studied, summarizes this behavior. As cholesterol concentration is increased, the average quadrupolar splitting increases, and the individual peaks broaden. However, at 22.5 mol % cholesterol, the individual lines become distinctly sharper and remain sharp at 25 mol %.

There is an interesting feature in the spectra at cholesterol concentrations of 20 mol % and higher, and at temperatures above about 25 °C. The signal due to the methyl groups is

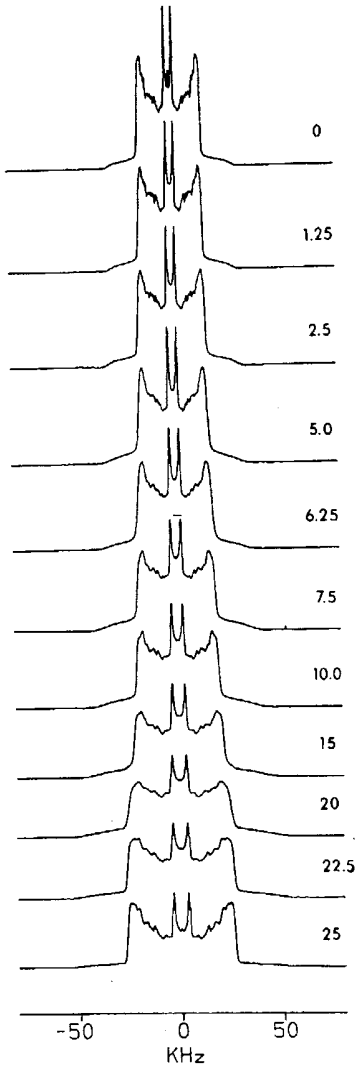

FIGURE 4: ^{2}H NMR spectra at 38 °C for all 12 cholesterol concentrations, labeled in mole percent.

split into two components of roughly equal area, as shown by the "de-Paked" spectra for 20 mol % cholesterol in Figure 5. This splitting must arise either from different average orientations of the terminal C–C bonds of the two chains or from different degrees of motional averaging of the two chains. The fact that the ratio of the two peaks is independent of cholesterol concentration and temperature indicates that the splitting is not the result of two lipid populations with different environments (e.g., two phases). In the pure phospholipid, there is significant disorder along the chain (i.e., via rapid gauche–trans isomerization) while in the presence of large amounts of cholesterol the chains are nearly in their all-trans conformation, as indicated by the value of the first moment, e.g., at 25 mol % at 38 °C, $M_1 = 9.75 \times 10^4$ s^{-1} compared to the value $M_1 = 5.53 \times 10^4$ s^{-1} at 0 mol %. To relieve packing problems at the center of the bilayer, it is possible that the last segment of the *sn*-2 chain, extending further into the bilayer than its neighbor, either adopts a slightly different average orientation when the chains are highly ordered or undergoes slightly different orientational averaging.

Figure 6 shows the spectrum as a function of cholesterol concentration at 36 °C, which is well below the chain-melting transition of pure DPPC-d_{62}. The spectrum is transformed from that characteristic of the $P_{\beta'}$ phase at low cholesterol concentrations to an axially symmetric line shape with sharply resolved features, reminiscent of the liquid-crystalline L_α phase, at 22.5 mol % cholesterol and above. In fact, there is a striking

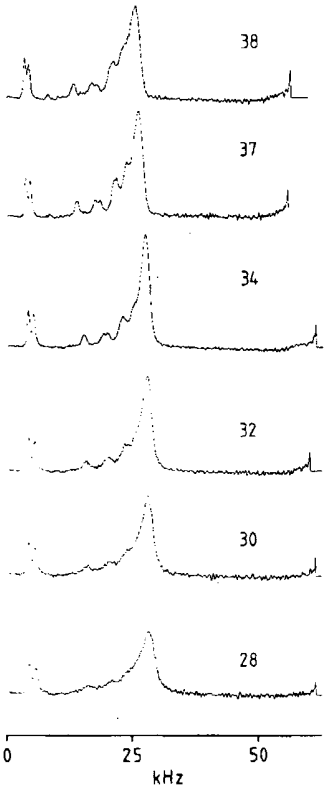

FIGURE 5: de-Paked ^{2}H NMR spectra of the 22.5 mol % cholesterol sample for selected temperatures, shown in degrees centigrade to the right of each spectrum.

similarity between the spectra for 22.5 and 25 mol % cholesterol in Figure 4, at 38 °C, and those at 36 °C in Figure 6. This is in sharp contrast with the large changes which occur between these two temperatures at low cholesterol concentrations.

For samples at these high cholesterol concentrations, the DSC traces, shown in Figure 2b, show a decrease in the amplitude and enthalpy of the sharp component while the broad high-temperature shoulder becomes more pronounced. At 22.5 mol % and above, the sharp component has vanished, and only a broad peak remains. Note that the DSC traces shown in Figure 2 have not been normalized and, in particular, that the trace at 22.5 mol % cholesterol was taken with a much larger sample than those at lower concentrations and is shown with an increased vertical expansion. Thus, the apparent areas in this figure should not be compared.

Moment Analysis of NMR Spectra. The moments of the symmetric ^{2}H NMR spectrum, $f(\omega)$, are defined by the equation:

$$M_n = \frac{1}{A}\int_0^\infty \omega^n f(\omega)\, d\omega \qquad (1)$$

where A is the area defined by

$$A = \int_0^\infty f(\omega)\, d\omega$$

and zero frequency is taken as the center of the spectrum. In this manner, we define the odd moments of these "half spectra" as well as the even moments (Davis, 1979, 1983).

The area of the spectrum, or its "zeroth moment", is proportional to the number of deuterons in the sample, in principle. In practice, there are deviations from this ideal due to a number of technical and experimental points: (i) the Q of the probe changes with temperature due to the temperature dependence of the dielectric constant of the sample; (ii) the

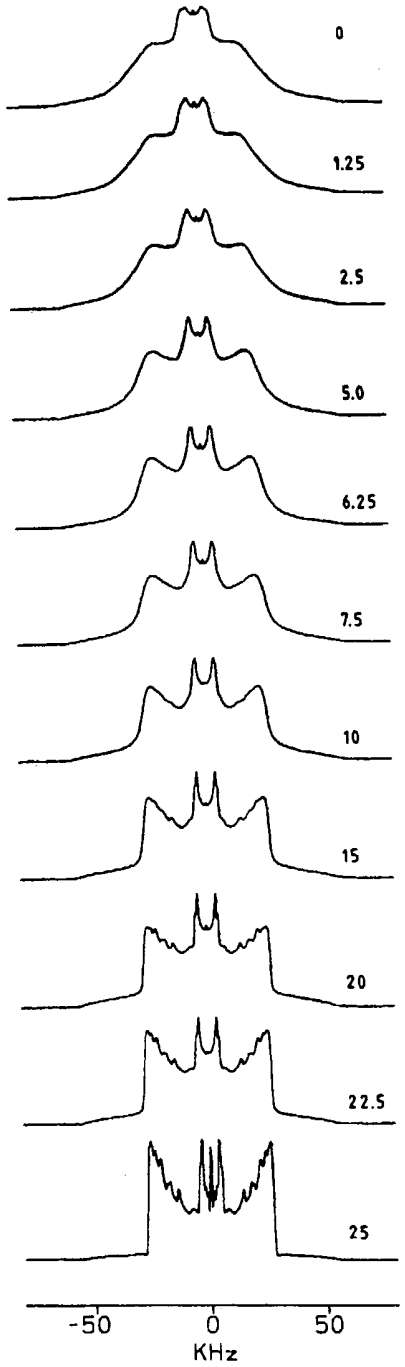

FIGURE 6: ^{2}H NMR spectra at 36 °C for all 12 cholesterol concentrations, labeled in mole percent.

sample's magnetization depends on temperature through the Boltzmann factor; (iii) since the quadrupolar echo technique is used, there is a decay of the magnetization from $t = 0$ to $t = 2\tau$, due to relaxation; and (iv) the area depends on the ratio of the quadrupolar splitting, ω_Q, and the rf field strength, ω_1 (Bloom et al., 1980). These last two points are especially important. The dependence of area on relaxation between $t = 0$ and $t = 2\tau$ can be used as a qualitative measure of the importance of slow motions (Blume & Griffin, 1982; Rice et al., 1987; Wittebort et al., 1987). Figure 7 shows the temperature and composition dependence of the area of the spectra (after normalization of the spectrum area of each sample to a value of 1.0 at 40 °C). The pure lipid, and samples with low cholesterol concentrations, shows a dramatic decrease in area if cooled through the chain-melting transition. This is consistent with observations on other lipid systems (Blume &

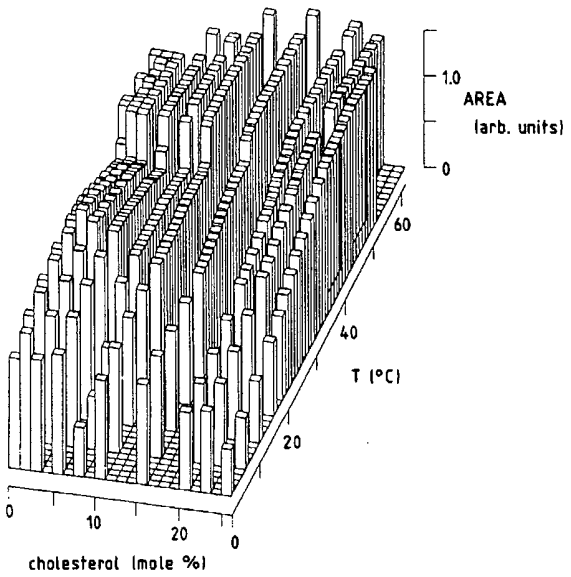

FIGURE 7: Histogram of the area of the ^2H NMR spectrum versus temperature and cholesterol composition. Areas are normalized to 1.0 at 40 °C for all samples.

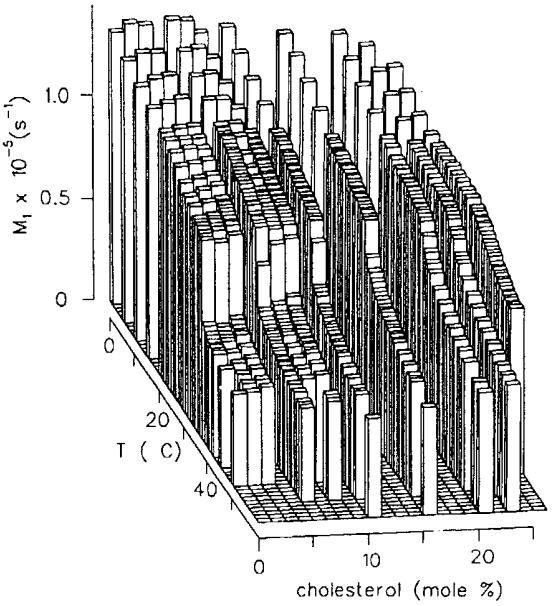

FIGURE 8: Histogram of the first moment of the ^2H NMR spectrum versus temperature and cholesterol composition. For clarity of presentation, the viewing angle for these data differs from that of the previous figure.

Griffin, 1982; R. G. Griffin, private communication). As cholesterol concentration is increased, this discontinuous change in area becomes smaller until at, and above, 20 mol % there is only a smooth gradual decrease as temperature is lowered. The dramatic decreases in area seen at the phase transition and at the lowest temperatures (near 0 °C) are due to the increased contribution of slow motions to relaxation, and to the effect of finite pulse width as ω_Q increases (Bloom et al., 1980).

The first moment, M_1, of an axially symmetric ^2H NMR powder pattern spectrum is proportional to the average quadrupolar splitting (Davis, 1977), even for perdeuteriated lipids. Figure 8 shows the temperature and composition dependence of M_1 for all 11 of the samples studied as a three-dimensional histogram. With this sort of plot, we can readily see some of the major features of the data. At temperatures above 37.75 °C, there is a steady increase in M_1 as cholesterol

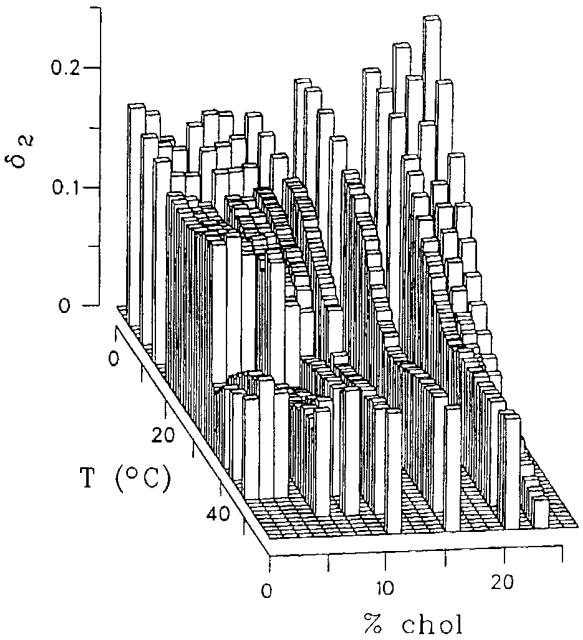

FIGURE 9: Histogram of the quantity $\delta_2 = (M_2/1.35M_1^2) - 1$ versus temperature and cholesterol composition.

concentration is increased. Conversely, at 20 °C, there is a steady decrease in M_1 with increasing cholesterol concentration. These results are consistent with earlier reports on the dependence of ^2H quadrupolar splittings on cholesterol concentration in DMPC, DPPC, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) (Haberkorn et al., 1977; Oldfield et al., 1978; Jacobs & Oldfield, 1979; Blume & Griffin, 1982; Wittebort et al., 1982). The obvious interpretation of these results is that the influence of cholesterol on lipid bilayers is to increase the average degree of orientational order in the fluid phase and to decrease the average order in the gel phase. It is interesting to note that at 36 °C $M_1 = 1.03\ (\pm 0.02) \times 10^4\ s^{-1}$ and is independent of cholesterol concentration at least between 0 and 25 mol %. The chain-melting transition of the pure phospholipid shows up as a nearly discontinuous change in M_1 by nearly a factor of 2. The discontinuity in M_1 gets smaller as cholesterol concentration is increased and eventually vanishes at high concentrations.

The shape of the ^2H NMR spectrum is sensitive to changes in average molecular orientational order. Above 38 °C, for all cholesterol concentrations studied, the motion is rapid enough that these molecular reorientations lead to an axially symmetric line shape. Below 38 °C, this is no longer true; here, the time scales for the molecular motions are in the intermediate regime—times of the order of 1/(width of the spectrum)—and the line shape is sensitive to details of the motions. Subtle changes which occur in the line shape often show up most clearly in the moments of the spectra. The function $\delta_2 = (M_2/1.35M_1^2) - 1$, which for axially symmetric ^2H NMR spectra is the mean squared width of the distribution of quadrupolar splittings, has been found to be strongly sensitive to the superposition of gel and liquid-crystalline phase spectra as is found in regions of two-phase coexistence (Davis, 1979; Davis et al., 1979a,b, 1980, 1983; Smith et al., 1979; Paddy et al., 1981). A three-dimensional histogram of δ_2 as a function of temperature and cholesterol concentration is shown in Figure 9. In the liquid-crystalline phase, the values of δ_2 are relatively small, indicative of a well-defined "plateau" in the quadrupolar splitting profile (Davis & Jeffrey, 1977; Davis, 1979), meaning that many of the deuterium positions near the head group have nearly the same quadrupolar splitting.

Substantial deviations from this value occur only near the end of the hydrocarbon chains. As cholesterol concentration is increased (for example, in the temperature range of from 45 to 50 °C), there is a sudden change in δ_2 from values in the range of 0.06–0.07 for samples with 6.25 mol % cholesterol or less to values in the range of 0.08–0.09 for samples with concentrations between 7 and 20 mol %. Above 20 mol %, the value of δ_2 again decreases to about 0.04 at 22 mol % and to less than 0.02 at 25 mol %. These changes correlate with the subtle changes in the broadening observed in the spectra in this region, as discussed above.

At the lowest cholesterol concentrations, δ_2 decreases rapidly as T is decreased toward the chain-melting transition at 37.75 °C. At the transition, for the 1.25 mol % cholesterol sample, there is an almost discontinuous increase in δ_2 from a value of 0.059 at 38 °C to a value of 0.179 at 37 °C. This is similar to the change seen in the pure DPPC-d_{62} sample. As cholesterol concentration is increased to 2.5, 5, and 6.25 mol %, the stepwise increase near 37.75 °C remains, but the total change in δ_2 between 37 and 38 °C decreases drastically, as shown in the figure.

At low cholesterol concentrations (2.5 mol % and below), δ_2 decreases gradually below 37 °C, reaching a local minimum at about 25 °C, correlating roughly with the position of the calorimetric pretransition near 28 °C. At 6 mol % cholesterol and higher, the value of δ_2 increases monotonically with decreasing temperature below 38 °C. This slow increase in δ_2 is at least partially due to the gradual slowing down in the rate of molecular reorientation as temperature is lowered.

^2H NMR Difference Spectroscopy. As we have seen near 37.75 °C for low cholesterol concentrations, the temperature/composition diagram of a two-component system can have regions of two-phase coexistence, consistent with Gibbs's phase rule. The boundaries of this two-phase region can be measured by a variety of techniques. Differential scanning calorimetry is a convenient method of observing a crossing of phase boundaries as temperature is varied provided that there is some enthalpy change associated with the phase change. In situations where the NMR spectra of the two phases are significantly different, such as at the gel to liquid-crystalline phase melting transition, then "nuclear magnetic calorimetry", i.e., watching for changes in the NMR spectra as temperature or concentration is changed, can be another fruitful method of mapping phase boundaries. In the case of the gel to liquid-crystalline phase transition, the change in the ^2H NMR spectrum is dramatic, as described above in reference to Figure 1.

Within a gel/liquid-crystalline two-phase region, at a temperature T and a sample composition x_A, our two-component system will have two kinds of domains. The first type of domain will be characteristic of a gel phase having a composition x_g given by the intersection of the isotherm at T and the "solidus", or lower phase boundary. The second type of domain will be characteristic of a fluid phase having a composition x_f given by the intersection of the isotherm at T and the "fluidus", or upper phase boundary. The partitioning of the sample between these two types of domains is given by the lever rule. For example, for a sample of composition x_i at a temperature T, the fluid fraction is given by

$$\alpha_i(T) = \frac{x_i - x_g(T)}{x_f(T) - x_g(T)} \tag{2}$$

and the gel fraction is $1 - \alpha_i(T)$. The amount of phospholipid in the fluid domains is determined from the relation

$$1 - x_i = \alpha_i(1 - x_f) + (1 - \alpha_i)(1 - x_g) \tag{3}$$

where $1 - x_i$ is the phospholipid concentration of the sample, and $1 - x_f$ and $1 - x_g$ are the phospholipid concentrations of the fluid- and gel-phase domains, respectively. The fraction of the phospholipid in the fluid-phase domains is then

$$f_i = \alpha_i \frac{1 - x_f}{1 - x_i} \tag{4}$$

and, of course, the gel phospholipid fraction is $1 - f_i$.

If the domains are sufficiently large and phospholipid diffusion is sufficiently slow that exchange of lipids between the two kinds of domains can be neglected on the ^2H NMR time scale (of the order of tens of microseconds to a millisecond in the present case), then the ^2H spectrum will be a superposition of two components. For gel/liquid-crystalline phase coexistence, there will be a broad component characteristic of the gel phase and a much narrower component due to lipid in the liquid-crystalline phase, as illustrated in Figure 1. If we consider the spectra of two samples of different compositions, x_A and x_B, taken at a temperature T such that both samples are in the same two-phase region of their temperature/composition plot, then we can write for the first sample that its spectrum is given by

$$S(x_A,T) = f_A S_f(x_f,T) + (1 - f_A) S_g(x_g,T) \tag{5}$$

while for the second sample

$$S(x_B,T) = f_B S_f(x_f,T) + (1 - f_B) S_g(x_g,T) \tag{6}$$

where $S_f(x_f,T)$ and $S_g(x_g,T)$ are the "end point" spectra coming from fluid-phase domains at end-point concentration x_f and from gel-phase domains at the other end-point concentration, x_g, and all spectra have been normalized to unit area. The major point here is that for two or more samples of different composition but at the same temperature within a two-phase region, the observed spectra are simply superpositions of the same two "end point" spectra characteristic of fluid- and gel-phase domains at the end-point concentrations. The only difference between the experimental spectra is the relative amount of each component. These fractions are given by the lever rule as described by eq 3 and 4.

Provided that we have at least two measurements at different compositions but the same temperature within a two-phase region, we can solve eq 5 and 6 for the end-point spectra

$$S_g(x_g,T) = \frac{1}{f_B - f_A}[f_B S(x_A,T) - f_A S(x_B,T)] \tag{7}$$

and

$$S_f(x_f,T) = \frac{1}{f_A - f_B}[(1 - f_B) S(x_A,T) - (1 - f_A) S(x_B,T)] \tag{8}$$

Clearly, we can generate the end-point spectra by subtracting a fraction of one of the normalized experimental spectra from the other.

If we let the concentration x_B correspond to the sample which has the higher fluid-phase content at T, then it will be possible to subtract a fraction K of $S(x_B,T)$ from $S(x_A,T)$ such that no fluid-phase component remains in the difference spectrum. This occurs when

$$K = \frac{\alpha_A(1 - x_B)}{\alpha_B(1 - x_A)} \tag{9}$$

Conversely, the fraction K' of $S(x_A,T)$, which must be subtracted from $S(x_B,T)$ to yield a difference spectrum with no gel-phase component, is given by

$$K' = \frac{(1 - \alpha_B)(1 - x_A)}{(1 - \alpha_A)(1 - x_B)} \tag{10}$$

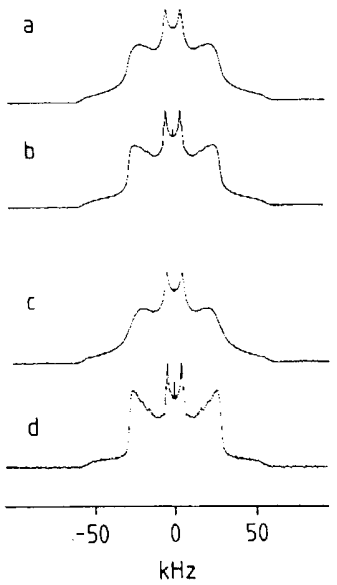

FIGURE 10: ^{2}H NMR difference spectroscopy, at 31 °C: (a) the spectrum of the 10 mol % cholesterol sample, normalized in area; (b) that of the 15 mol % sample, also normalized; (c) the spectrum in "a" minus 0.34 times that in "b", renormalized; and (d) the spectrum in "b" minus 0.59 times the spectrum in "a", renormalized.

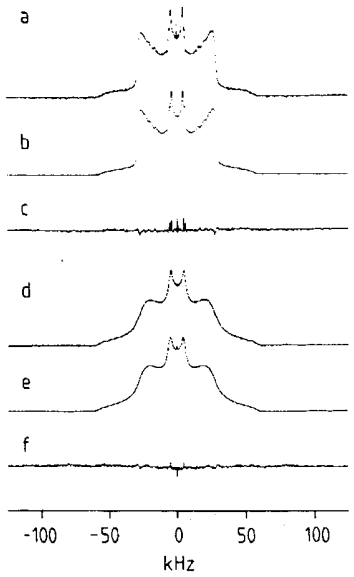

FIGURE 11: Comparison of the end-point difference spectra of Figure 10 with spectra from samples with cholesterol compositions near the end-point concentrations: (a) the end-point difference spectrum from Figure 10d, normalized in area; (b) the spectrum at 31 °C of the 22.5 mol % cholesterol sample, also normalized; (c) the difference between the spectrum in "a" and that in "b"; (d) the end-point difference spectrum from Figure 10c, normalized; (e) the spectrum at 31 °C of the 7.5 mol % cholesterol sample, also normalized; (f) the difference between the spectrum in "d" and that in "f".

Finally, from eq 2, 4, 9, and 10, we can solve for the end-point concentrations:

$$x_g = \frac{(1 - x_B)x_A - K(1 - x_A)x_B}{(1 - x_B) - K(1 - x_A)} \quad (11)$$

and

$$x_f = \frac{(1 - x_A)x_B - K'(1 - x_B)x_A}{(1 - x_A) - K'(1 - x_B)} \quad (12)$$

This procedure will be valid for any two-phase coexistence region (not only for gel and fluid phases), provided that the basic assumption of slow exchange of phospholipid between domains holds and that the two phases have end-point spectra which are sufficiently different that one can carry out the subtraction procedure. This method has been used successfully in studies of lipid/peptide mixtures (Huschilt et al., 1985; Morrow et al., 1985; Morrow & Davis, 1988). The equations used in these earlier studies were based on the assumption of small peptide concentration whereas no such assumption can be made in the cholesterol/DPPC case.

The spectra of Figure 1 clearly demonstrate two-phase coexistence at cholesterol concentrations below 7 mol %. For a narrow range of temperatures, we see a coexistence of fluid- and gel-phase components in the spectra. Unfortunately, even though we have studied five different cholesterol concentrations in this region (0, 1.25, 2.5, 5, and 6.25 mol %), the two-phase region is so narrow that the uncertainties in temperature (about 0.25 °C) make it unlikely that we could gain anything by applying difference spectroscopy in this region. In this situation, we can map out the phase boundaries sufficiently well simply by watching for the appearance or disappearance of one of the components in the spectrum.

On the other hand, for concentrations from 7 to 22 mol % cholesterol, at temperatures between about 25 and 37 °C, the spectra do not appear to be obvious superpositions of two distinguishable end-point spectra. The series of spectra as a function of cholesterol concentration at 36 °C, shown in Figure 6, at first suggests only a rather gradual transition from the characteristic $P_{\beta'}$-phase spectrum at low cholesterol concentration to the axially symmetric spectrum characteristic of the highest cholesterol concentrations. Casual inspection proves to be deceiving, however, since in this region the experimental spectra can be nicely separated into their two components by spectral subtraction.

To illustrate, Figure 10a,b shows normalized spectra at 10 and 15 mol % cholesterol at 31 °C. While neither spectrum is an obvious superposition of two independent components, subtracting 59% of the 10 mol % spectrum from that of the 15 mol % sample yields the axially symmetric difference spectrum shown in Figure 10d. The value $K' = 0.593$ gives the end-point concentration $x_\beta = 0.214$. This difference spectrum, now renormalized in Figure 11a, is compared with the normalized experimental spectrum obtained at $x = 0.225$ and 31 °C in Figure 11b. In Figure 11c is the difference between the spectra in "a" and "b". Clearly, the end-point spectrum corresponding to $x_\beta = 0.214$, obtained by difference spectroscopy, and the spectrum of the 22.5 mol % sample are nearly identical, the biggest difference occurring at the sharp methyl group resonances where one can expect to have difficulty even under ideal circumstances.

If we subtract 34.2% of the spectrum of Figure 10b from that of Figure 10a, then we obtain the end-point spectrum shown as Figure 10c, corresponding to an end-point concentration $x_g = 0.072$. This difference spectrum, after normalization (Figure 11d), is compared to the experimental spectrum obtained for the 7.5 mol % sample at 31 °C in Figure 11e. The difference between the end-point spectrum and the 7.5 mol % spectrum is shown in Figure 11f. Just as we found at the "β"-phase boundary, the agreement between the two is remarkable. Comparison of these end-point spectra with experimental spectra at slightly different concentrations, e.g., in the latter case, with the spectrum for the 6.25 mol % sample, shows large differences. It is difficult to determine precisely how much of a spectrum like that in Figure 10b should be subtracted from one like that in Figure 10a, and this uncertainty is reflected in the error in determining the concentration at the phase boundary. In doing the subtractions, we look for the appearance of inflection points near the shoulders of the

spectrum (near ±30 kHz) and for the appearance or disappearance of the sharp features characteristic of the β phase. Fortunately, comparison of the end-point spectrum with experimental spectra for samples with concentrations near the phase boundaries turns out to be quite sensitive.

Our initial choice of sample concentrations resulted in our having only two concentrations within this two-phase region which could be used for these linear decompositions (the sample at 20 mol % cholesterol lies too close to the β-phase boundary for us to be able to use it); otherwise, we could have formed various pairwise decompositions and compared the end points obtained as in Huschilt et al. (1985). Nonetheless, the results of Figures 10 and 11 show conclusively that below 37 °C, between about 7 and 22 mol % cholesterol, we are in a two-phase coexistence region. Spectral subtractions from 30 to 37 °C all yield similar results, with end-point spectra very similar to the experimental spectra at 7.5 and 22.5 mol %, at corresponding temperatures.

At 28 °C, we begin to have difficulty so that we can extract only the gel-phase end point at 27 and 26 °C, but can no longer determine the β-phase end point. At these lower temperatures, the spectra even at high cholesterol concentrations (22.5 and 25 mole %) are no longer axially symmetric fast-limit spectra. Without the distinct differences between spectra in the two phases, it becomes impossible to define phase boundaries by difference spectroscopy. Similar problems are encountered at temperatures above 37 °C at cholesterol concentrations between 7.5 and 20 mol %. Here, we expect a coexistence of liquid-crystalline and β-phase domains. At 22.5 and 25 mol %, the spectra do not change appreciably as the temperature is increased from 36 to 39 °C, there being only a small decrease in the observed splittings. The changes in the spectra at 38 °C, shown in Figure 4, suggest a two-phase region where the domain size is so small that lipids can diffuse from one domain to another during the spectroscopic time scale. Under these conditions, we cannot expect the subtraction procedure to work, and indeed it does not. A spectroscopic technique with a considerably different time scale, for example, electron paramagnetic resonance, should show evidence for two-phase coexistence in this region. The EPR work of Shimshick and McConnell (1973) on DMPC/cholesterol and DPPC/cholesterol mixtures and of Recktenwald and McConnell (1981) on DMPC/cholesterol mixtures shows evidence of two-phase coexistence both above and below the main chain-melting transition of the pure phospholipid system.

The temperature and composition dependence of the area of the spectrum (Figure 7) strongly supports our contention that there is a two-phase coexistence region below 37 °C between about 7.5 and 20 mol % cholesterol. The discontinuous decrease in area at the chain-melting phase transition accompanies the transition from fluid to gel phase. There is no change over that temperature range for samples within the β phase. As cholesterol concentration is increased from 7.5 to 20 mol %, the size of the discontinuous change in area decreases from about 30% of the total area at 7.5 mol % cholesterol, which is essentially the same as that of the pure phospholipid, to 25% for the 10 mol % sample, 14% for the 15 mol % sample, and, finally, to 0% at 20 mol % cholesterol. This is essentially a linear dependence on the amount of gel-phase lipid in the two-phase region, as expected.

Partial Phase Diagram of DPPC/Cholesterol. So far, we have identified two regions of two-phase coexistence. The first is a region of gel and liquid-crystalline phase coexistence, lying in a narrow temperature range just below the melting point of pure DPPC/water, at 37.75 °C, and covering the cholesterol

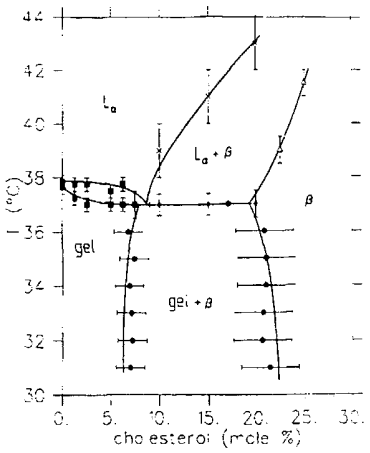

FIGURE 12: Partial phase diagram for cholesterol/DPPC mixtures. The phases are labeled as follows: L_α, the fluid or liquid-crystalline phase; G, the gel phase; β, the high cholesterol content phase characteristic of biological membranes. The squares (■) are determined by inspection of spectra such as those in Figure 1, the circles (●) are from difference spectroscopy, the diamonds (♦) show the three-phase line determined by the temperature of the sharp peak in the DSC traces, the crosses (×) are from the upper limit of the broad component of the DSC traces, and the triangles (△) are from the sharpening of the resonances at high cholesterol concentrations. The solid lines simply delineate the phase boundaries as described above.

concentration range from 0 to about 7 mol %. The boundaries of this two-phase region have been estimated from both NMR and DSC and are shown in Figure 12, the solid squares being given by the NMR spectra. The second two-phase region lies between about 7.5 and 22 mol % cholesterol, below 37 °C. Here we found a coexistence of gel and "β" phases, and the phase boundaries, shown as solid circles in Figure 12, were found by using difference spectroscopy. At about 37 °C, there is a three-phase line where liquid-crystalline, gel, and "β" phases are all in coexistence at a single temperature, for concentrations between about 7.5 and 20 mol %. This three-phase line corresponds to the melting of the gel-phase component into a liquid-crystalline phase component which shows up as a sharp peak in the DSC scan traces shown in Figure 2. The temperature of this sharp DSC component is shown by the solid diamonds in Figure 12.

A three-phase line cannot form a boundary between a two-phase region (e.g., the gel/"β" region) and a one-phase region, the liquid-crystalline or L_α phase. Thus, we expect to find another two-phase region above this three-phase line, extending from the intersection of the three-phase line with the L_α/gel fluidus, or upper two-phase line, to its end (near 20 mol % cholesterol). The shoulder on the high-temperature side of the sharp peak near 37 °C in the DSC traces extends to the upper boundary of this new two-phase region, allowing us to estimate the position of the boundary between the L_α-phase and the L_α/"β"-phase two-phase region. This boundary is indicated by crosses in Figure 12. It should be noted, however, that if we are near a critical mixing point, the DSC traces will tend to extend beyond the phase boundaries and will overestimate the width of the two-phase coexistence region. The boundary between the "β" phase and this two-phase region is determined by examination of the ^{2}H NMR spectra at high cholesterol concentrations. For example, for the 22.5 mol % sample, as temperature is varied from 28 to 38 °C, there is little change in the spectrum, only a gradual decrease in the splittings. The individual sharp peaks in the spectrum remain well-defined until we reach 39 °C where all of the peaks broaden considerably. At 25 mol %, this broadening occurs between 41 and 42 °C. These points are

shown as open triangles in Figure 12.

Within this L_α/β two-phase region, the spectra of both the L_α-phase and the "β"-phase domains are similar, displaying the axial symmetry of the molecular motions. The primary difference between the two spectra is the average value of the quadrupolar splittings. For example, at 38 °C, M_1 changes from about 7.1×10^4 s^{-1} at 7.5 mol % to 9.6×10^4 s^{-1} at 22.5 mol % cholesterol, an increase of about 35%. The largest quadrupolar splitting changes from 37.4 to 50.8 kHz under the same conditions. This difference in quadrupolar splittings can be used to estimate a spectroscopic time scale of about 75 μs. The diffusion constant for cholesterol/DPPC mixtures is about 2×10^{-8} cm^2/s and is relatively independent of cholesterol concentration at temperatures above the pure lipid-phase transition (Rubenstein et al., 1979; Kuo & Wade, 1979; Lindblom et al., 1981). This diffusion rate is similar in both the L_α and "β" phases. We estimate that a phospholipid can diffuse a root mean squared distance of about 25 nm during this NMR time scale. If the domains in this two-phase region are smaller than, or of the order of, this characteristic distance, then the NMR experiment will see an averaged spectrum, and the difference spectroscopy technique will not be able to separate the spectrum into two components. The broadening of the spectrum which occurs within this region is suggestive of this exchange averaging.

Conclusion

Cholesterol causes dramatic changes in membrane molecular order and dynamics in the fluid (L_α) and gel ($P_{\beta'}$ and $L_{\beta'}$) phases as well as introducing a new thermodynamic phase at high cholesterol concentrations, the β phase. The most obvious change in the L_α phase (the thermodynamically stable phase found at low cholesterol concentrations above the chain-melting transition), seen by ^{2}H NMR experiments, is the large increase in average orientational order of the lipid chains, expressed by M_1. This result is in agreement with earlier ^{2}H NMR work on DPPC/cholesterol (Haberkorn et al., 1977) and ^{2}H and ^{13}C NMR on DMPC/cholesterol mixtures (Jacobs & Oldfield, 1979; Oldfield et al., 1978; Blume & Griffin, 1982; Wittebort et al., 1982; Cornell & Keniry, 1983). This increase in chain order is accompanied by (or results in) an increase in bilayer thickness (Lecuyer & Dervichian, 1969; Schwartz & Paltauf, 1976; Stockton & Smith, 1976; McIntosh, 1978; Hui & He, 1983). At the same time, there seems to be little change in the lateral diffusion rate of unsaturated lecithins (Lindblom et al., 1981) or fluorescent probes (Rubenstein et al., 1979; Alecio et al., 1982), but there is apparently a significant increase in diffusion rate for DPPC/cholesterol mixtures (Kuo & Wade, 1979) as cholesterol concentration is increased. In spite of the large increase in chain order, dielectric relaxation measurements (Shepherd & Buldt, 1979; Henze, 1980) suggest a decrease in the correlation time for head-group reorientation as cholesterol concentration is increased, and ^{2}H NMR of head-group-labeled DPPC (Brown & Seelig, 1978) suggests increased motional freedom due to a looser packing in the head-group region.

The change in hydrocarbon chain order within the gel phase (i.e., the thermodynamically stable phase at low cholesterol concentrations and at temperatures just below the chain-melting transition), as cholesterol is added, is small because in the gel phase the chains are already largely all-trans. The calorimetry and ^{2}H NMR results indicate that the pretransition is suppressed at concentrations above about 6 mol % cholesterol. Unfortunately, we could not follow the phase boundary between the $P_{\beta'}$ and $L_{\beta'}$ phases as cholesterol concentration was varied. X-ray diffraction experiments indicating a dramatic increase in the bilayer repeat spacing as cholesterol concentration is raised from 0 to 5 mol % at temperatures within the gel-phase region of DMPC/cholesterol mixtures (McIntosh, 1978; Hui & He, 1983) have been interpreted as being due to a reduction in the tilt angle. The reduction is more pronounced at lower temperatures. This suggests that at about 7.5 mol % cholesterol at temperatures below the chain-melting temperature, the system either is in the L_β phase rather than in either the $P_{\beta'}$ or the $L_{\beta'}$ phase, or at least that the tilt angle is significantly reduced. The rate of lateral diffusion in the gel phase increases with cholesterol content (Rubenstein et al., 1979; Kuo & Wade, 1979; Alecio et al., 1982) even for concentrations less than about 7.5 mol %. As is the case for the L_α phase, we have little information on the details of the influence of cholesterol on phospholipid dynamics in the gel phase.

The β phase, found at concentrations greater than about 22 mol % cholesterol, is of fundamental interest because it seems to be the phase characteristic of biological membranes containing large amounts of cholesterol. In ^{2}H NMR studies of human erythrocyte ghosts (Davis et al., 1979a; Maraviglia et al., 1982) and of *Acholeplasma laidlawii* grown on cholesterol-rich media (Davis et al., 1980; Rance et al., 1982), the membranes were found to be in a relatively "fluid" state where the ^{2}H NMR spectra showed rapid axially symmetric motion of the lipids, but where the chains were highly ordered becoming nearly all-trans at lower temperatures. The DPPC/cholesterol mixtures at high cholesterol concentrations give analogous NMR spectra. The term "fluid" is taken to mean a state characterized by rapid axially symmetric reorientation of the phospholpids and lateral diffusion rates comparable to those found in the L_α phase (Rubenstein et al., 1979; Alecio et al., 1982).

The phospholipid chains are much more highly ordered in the β phase than in the L_α phase (Stockton & Smith, 1976; Haberkorn et al., 1977; Oldfield et al., 1978; Jacobs & Oldfield, 1979; Recktenwald & McConnell, 1981; Presti & Chan, 1982; Wolber & Hudson, 1981; Kutchai et al., 1983). M_1 is typically about 75% larger at 25 mol % cholesterol than its value for the pure phospholipid in the fluid phase at corresponding temperatures. The values of M_1 in the β phase are actually comparable to the values for the gel phase of the pure phospholipid. Even so, all of these systems remain in an essentially "fluid" state even at temperatures well below the hydrocarbon chain-melting temperature.

^{13}C NMR experiments suggest that, at high cholesterol concentrations, essentially the only motion experienced by the phospholipid carbonyl groups is a rapid axially symmetric reorientation (Cornell & Keniry, 1983). Fluorescence depolarization studies of cholesterol/unsaturated PC mixtures found that the rate of rotational diffusion of the fluorescent probe molecules was greater in the presence of 20 mol % cholesterol than in the pure lipid's L_α phase (van Ginkel et al., 1986). The total d spacing measured by X-ray diffraction is nearly independent of cholesterol concentration and temperature within the β phase (Hui & He, 1983) and has a value which is intermediate between that found in the gel phase and that of the L_α phase. Micromechanical measurements made by stretching the surface of single-bilayer vesicles (Needham et al., 1988) find that the area compressibility modulus is roughly 4 times as large at 33 mol % cholesterol in DMPC as it is for DMPC along in the L_α phase, but only slightly smaller than the value found for the gel phase of DMPC.

The characteristics of the β phase can be summarized as follows: (i) the bilayer has a lateral diffusion rate which is

comparable to that of the L_α phase; (ii) axial molecular reorientation is rapid, having rotational diffusion rates comparable to those of the L_α phase; (iii) the phospholipid chains are highly ordered; (iv) the bilayer is thicker than in the L_α phase but thinner than in the gel phase; and (v) a greater tension is required to stretch the bilayer in the β phase than in the L_α phase. The effect of cholesterol in phospholipid bilayers is to increase the thickness and "strength" of the bilayer while maintaining the "fluid" environment conducive to high lateral mobility. This modification of the physical characteristics of the bilayer may be one of its major functional roles in the membrane (Bloom & Mouritsen, 1988).

We have identified three separate two-phase regions: (i) a narrow region of coexistence of L_α and gel phases between 0 and 7.5 mol % cholesterol; (ii) a gel-phase/β-phase region below about 37 °C between 7.5 and about 22.5 mol % cholesterol; and (iii) an L_α/β-phase coexistence region above 37 °C starting at the eutectic point, somewhere between 7.5 and 10 mol %, and ending near 22.5 mol %. Because it is so narrow, about 1 °C wide, the L_α/gel coexistence region has not been previously discussed in the literature even though its existence is expected from the fundamental principles of two-component mixtures (Guggenheim, 1952). The second two-phase region, the gel/β-phase coexistence, has been seen in neutron scattering experiments on DMPC/cholesterol mixtures where phase boundaries at 8 and 24 mol % cholesterol were reported (Knoll et al., 1985) and in EPR studies on the same system (Recktenwald & McConnell, 1981). The third two-phase region, of L_α/β-phase coexistence, is suggested by DSC, fluorescence (Lentz et al., 1980), and EPR data (Shimshick & McConnell, 1973; Recktenwald & McConnell, 1981). While ^2H NMR difference spectroscopy enabled us to determine the phase boundaries of the gel/β-phase region, the high rate of lateral diffusion and/or the small domain size in the L_α/β-phase region prevented us from using that technique in the latter case.

The boundary between the β-phase and the gel/β-phase coexistence region, occurring near 20 mol % cholesterol, has frequently been drawn as a vertical line (Recktenwald & McConnell, 1981; Mortensen et al., 1938). The error in the end-point determinations is too large for us to be able to accurately determine the slope of this boundary from such a narrow temperature range. Unpublished NMR work on mixtures of cholesterol and a series of disaturated PC's (R. G. Griffin, private communication) indicates that this boundary is not vertical but slopes to the right (toward higher cholesterol concentrations).

Ipsen et al. (1987) have developed a theoretical model which treats the DPPC/cholesterol mixtures as a pseudoternary system where the three components are chain-disordered phospholipid, chain-ordered phospholipid, and cholesterol. Allowing these three types of molecules to have different pairwise interaction energies results in a phase diagram very similar to that reported here (Ipsen et al., 1987). This modeling of our DPPC/cholesterol phase diagram has predicted that the L_α/β-phase coexistence region is really a miscibility gap and, therefore, that there is a critical mixing point (Ipsen et al., 1987). The broad maximum in the temperature dependence of the specific heat at cholesterol concentrations greater than about 22.5 mol %, as measured by DSC and shown by the last trace in Figure 2, does not correspond to the crossing of any phase boundaries or two-phase regions. Near such a critical point, one expects to observe just such a maximum in the specific heat (Morrow et al., 1985; Morrow & Whitehead, 1988).

Further theoretical and experimental studies of the phase equilibria of phospholipid/sterol model systems will help lead to a fundamental understanding of the role of cholesterol in membranes. Quantitative comparison of calorimetric measurements with theoretical models will allow us to compare the interaction energies of phospholipids with various sterols and may enable us to identify the specific features of cholesterol which make it such an effective modulator of membrane physical chracteristics.

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References

Alecio, M. R., Golan, D. E., Veatch, W. R., & Rando, R. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5171–5174.

Alecio, M. R., Miller, A., & Watts, A. (1985) *Biochim. Biophys. Acta 815*, 139–142.

Bienvenue, A., Bloom, M., Davis, J. H., & Devaux, P. F. (1982) *J. Biol. Chem. 257*, 3032–3038.

Bloom, M., & Mouritsen, O. G. (1988) *Can. J. Chem. 66*, 706–712.

Bloom, M., Davis, J. H., & Valic, M. I. (1980) *Can. J. Phys. 58*, 1510–1517.

Blume, A., & Griffin, R. G. (1982) *Biochemistry 21*, 6230–6242.

Brainard, J. R., & Cordes, E. H. (1981) *Biochemistry 20*, 4607–4617.

Brown, M. F., & Seelig, J. (1978) *Biochemistry 17*, 381–384.

Calhoun, W. I., & Shipley, G. G. (1979) *Biochemistry 18*, 1717–1722.

Chapman, D. (1975) *Q. Rev. Biophys. 8*, 185–235.

Copeland, B. R., & McConnell, H. M. (1980) *Biochim. Biophys. Acta 599*, 95–109.

Cornell, B. A., & Keniry, M. (1983) *Biochim. Biophys. Acta 732*, 705–710.

Cornell, B. A., Chapman, D., & Peel, W. E. (1979) *Chem. Phys. Lipids 23*, 223–237.

Cornell, B. A., Davenport, J. B., & Separovic, F. (1982) *Biochim. Biophys. Acta 689*, 337–345.

Cullis, P. R., Hope, M. J., de Kruijff, B., Verkleij, A. J., & Tilcock, C. P. S. (1985) in *Phospholipids and Cellular Regulation* (Kuo, J. F., Ed.) Vol. 1, pp 1–59, CRC Press, Boca Raton, FL.

Dahl, C. E. (1981) *Biochemistry 20*, 7158–7161.

Davis, J. H. (1979) *Biophys. J. 27*, 339–358.

Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.

Davis, J. H. (1988) in *Physics of NMR Spectroscopy in Biology and Medicine, Proceedings of the 100th International School of Physics "Enrico Fermi"* (Maraviglia, B., Ed.) pp 302–312, Society of Italiana di Fisica, Bologna, Italy.

Davis, J. H., & Jeffrey, K. R. (1977) *Chem. Phys. Lipids 20*, 87–104.

Davis, J. H., Maraviglia, B., Weeks, G., & Godin, D. V. (1979a) *Biochim. Biophys. Acta 550*, 362–366.

Davis, J. H., Nichol, C. P., Weeks, G., & Bloom, M. (1979b) *Biochemistry 18*, 2103–2112.

Davis, J. H., Bloom, M., Butler, K. W., & Smith, I. C. P. (1980) *Biochim. Biophys. Acta 597*, 447–491.

Davis, J. H., Clare, D. M., Hodges, R. S., & Bloom, M. (1983) *Biochemistry 22*, 5298–5305.

Davis, P. J., & Keough, K. M. W. (1983) *Biochemistry 22*, 6334–6340.
Deinum, G., van Langen, H., van Ginkel, G., & Levine, Y. K. (1988) *Biochemistry 27*, 842–860.
de Kruijff, B. (1978) *Biochim. Biophys. Acta 506*, 173–182.
de Kruijff, B., Cullis, P. R., Radda, G. K., & Richards, R. E. (1976) *Biochim. Biophys. Acta 419*, 411–424.
Delmelle, M., Butler, K. W., & Smith, I. C. P. (1980) *Biochemistry 19*, 698–704.
Demel, R. A., & de Kruijff, B. (1976) *Biochim. Biophys. Acta 457*, 109–132.
El-Sayed, M. Y., Guion, T. A., & Fayer, M. D. (1986) *Biochemistry 25*, 4825–4832.
Estep, T. N., Mountcastle, D. B., Biltonen, R. L., & Thompson, T. E. (1978) *Biochemistry 17*, 1984–1989.
Fahey, P. F., & Webb, W. W. (1978) *Biochemistry 17*, 3046–3053.
Franks, N. P. (1976) *J. Mol. Biol. 100*, 345–358.
Gally, H. U., Seelig, A., & Seelig, J. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1447–1450.
Genz, A., Holzwarth, J. F., & Tsong, T. Y. (1986) *Biophys. J. 50*, 1043–1051.
Gershfeld, N. L. (1978) *Biophys. J. 22*, 469–488.
Ghosh, R., & Seelig, J. (1982) *Biochim. Biophys. Acta 691*, 151–160.
Gruen, D. W. R. (1982) *Chem. Phys. Lipids 30*, 105–120.
Guggenhem, E. A. (1952) *Mixtures*, Clarendon Press, Oxford.
Gupta, C. M., Radakrishnan, R., & Khorana, H. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4315–4319.
Haberkorn, R. A., Griffin, R. G., Meadows, M. D., & Oldfield, E. (1977) *J. Am. Chem. Soc. 99*, 7353–7355.
Henze, R. (1980) *Chem. Phys. Lipids 27*, 165–175.
Hildenbrand, K., & Nicolau, C. (1979) *Biochim. Biophys. Acta 553*, 354–377.
Hinz, H. J., & Sturtevant, J. M. (1972) *J. Biol. Chem. 247*, 3697–3700.
Hui, S. W., & He, N.-B. (1983) *Biochemistry 22*, 1159–1164.
Huschilt, J. C., Hodges, R. S., & Davis, J. H. (1985) *Biochemistry 24*, 1377–1386.
Imaizumi, S., & Hatta, I. (1984) *J. Phys. Soc. Jpn. 53*, 4476–4487.
Ipsen, J. H., Karlstrom, G., Mouritsen, O. G., Wennerstrom, H., & Zuckermann, M. H. (1987) *Biochim. Biophys. Acta 905*, 162–172.
Jacobs, R., & Oldfield, E. (1979) *Biochemistry 18*, 3280–3285.
Jahnig, F. (1981a) *Biophys. J. 36*, 329–345.
Jahnig, F. (1981b) *Biophys. J. 36*, 347–357.
Janiak, M. J., Small, D. M., & Shipley, G. G. (1976) *Biochemistry 15*, 4575–4580.
Jarrell, H. C., Byrd, R. A., & Smith, I. C. P. (1981) *Biophys. J. 34*, 451–463.
Kar, L., Ney-Igner, E., & Freed, J. H. (1985) *Biophys. J. 48*, 569–595.
Kawato, S., Kinosita, K., Jr., & Ikegami, A. (1978) *Biochemistry 17*, 5026–5031.
Knoll, W., Schidt, G., Ibel, K., & Sackmann, E. (1985) *Biochemistry 24*, 5240–5246.
Kuo, A.-L., & Wade, C. G. (1979) *Biochemistry 18*, 2300–2308.
Kutchai, H., Chandler, L. H., & Zavoico, G. B. (1983) *Biochim. Biophys. Acta 736*, 137–149.
Ladbrooke, B. D., Williams, R. M., & Chapman, D. (1968) *Biochim. Biophys. Acta 150*, 333–340.
Lee, a. G. (1977) *Biochim. Biophys. Acta 472*, 285–344.
Lentz, B. R. Barrow, D. A., & Hoechli, M. (1980) *Biochemistry 19*, 1943–1954.
Lindblom, G., Johansson, L. B.-A., & Arvidson, G. (1981) *Biochemistry 20*, 2204–2207.
Mabrey, S., Mateo, P. L., & Sturtevant, J. M. (1978) *Biochemistry 17*, 2464–2468.
Maraviglia, B., Davis, J. H., Bloom, M., Westerman, J., & Wirtz, K. W. A. (1982) *Biochim. Biophys. Acta 686*, 137–140.
Marcelja, S. (1974) *Biochim. Biophys. Acta 367*, 165–176.
McElhaney, R. N. (1982) *Chem. Phys Lipids 30*, 229–259.
McIntosh, T. J. (1978) *Biochim. Biophys. Acta 513*, 43–58.
Melchior, D. L., Scavitto, F. J., & Steim, J. M. (1980) *Biochemistry 19*, 4828–4834.
Morrow, M. R., & Davis, J. H. (1988) *Biochemistry 27*, 2024–2032.
Morrow, M. R., & Whitehead, J. P. (1988) *Biochim. Biophys. Acta 941*, 271–277.
Morrow, M. R., Huschilt, J. C., & Davis, J. H. (1985) *Biochemistry 24*, 5396–5406.
Morrow, M. R., Davis, J. H., Sharom, F. J., & Lamb, M. P. (1986) *Biochim. Biophys. Acta 858*, 13–20.
Mortensen, K., Pfeiffer, W., Sackmann, E., & Knoll, W. (1988) *Biochim. Biophys. Acta 945*, 221–245.
Mouritsen, O. G., Boothroyd, A., Harris, R., Jan, N., Lookman, T., MacDonald, L., Pink, D. A. & Zuckermann, M. (1983) *J. Chem. Phys. 79*, 2027–2041.
Needham, D., McIntosh, T. J., & Evans, E. (1988) *Biochemistry 27*, 4668–4673.
Offringa, J. C. A., Plekkenpol, R., & Crommelin, D. J. A. (1987) *J. Pharm. Sci. 76*, 821–824.
Oldfield, E., & Chapman, D. (1971) *Biochem. Biophys. Res. Commun. 43*, 610–616.
Oldfield, E., Chapman, D., & Derbyshire, W. (1971) *FEBS Lett. 16*, 102–104.
Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978) *Biochemistry 17*, 2727–2740.
Opella, S. J., Yesinowski, J. P., & Waugh, J. S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3812–3815.
Owicki, J. C., & McConnell, H. M. (1980) *Biophys. J. 30*, 381–398.
Paddy, M. R., Dahlquist, F. W., Davis, J. H., & Bloom, M. (1981) *Biochemistry 20*, 3152–3162.
Phillips, M. C. (1972) *Prog. Surf. Membr. Sci. 5*, 139–221.
Pink, D. A., & Carroll, C. E. (1978) *Phys. Lett. 66a*, 157–160.
Pink, D. A., & Chapman, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1542–1546.
Presti, F. T., & Chan, S. I. (1982) *Biochemistry 21*, 3821–3830.
Rance, M., Jeffrey, K. R., Tulloch, A. P., Butler, K. W., & Smith, I. C. P. (1982) *Biochim. Biophys. Acta 688*, 101–200.
Rand, R. P., Parsegian, V. A., Henry, J. A. C., Lis, L. J., & McAlister, M. (1980) *Can. J. Biochem. 58*, 959–968.
Recktenwald, D. J., & McConnell, H. M. (1981) *Biochemistry 20*, 4505–4510.
Rice, D. M., Meinwald, Y. C., Scheraga, H. A., & Griffin, R. G. (1987) *J. Am. Chem. Soc. 109*, 1636–1640.
Rubenstein, J. L. R., Smith, B. A., & McConnell, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 15–18.
Rubenstein, J. L. R., Owicki, J. C., & McConnell, H. M. (1980) *Biochemistry 19*, 569–573.
Schreier-Mucillo, S., Marsh, D., Dugas, H., Schneider, H., & Smith, I. C. P. (1973) *Chem. Phys. Lipids 10*, 11–27.

Schwartz, E. T., Paltauf, F., & Laggner, P. (1976) *Chem. Phys. Lipids 17*, 423-434.
Scott, H. L., & Cherng, S.-L. (1978) *Biochim. Biophys. Acta 510*, 209-215.
Shepherd, J. C. W., & Buldt, G. (1979) *Biochim. Biophys. Acta 558*, 41-47.
Shimshick, E. J., & McConnell, H. M. (1973) *Biochem. Biophys. Res. Commun. 53*, 446-451.
Smith, I. C. P., Butler, K. W., Tulloch, A. P., Davis, J. H., & Bloom, M. (1979) *FEBS Lett. 100*, 57-61.
Smutzer, G. (1988) *Biochim. Biophys. Acta 946*, 270-280.
Smutzer, G., & Yeagle, P. L. (1985) *Biochim. Biophys. Acta 814*, 274-280.
Stamatoff, J., Feuer, B., Guggenheim, H. J., Tellez, G., & Yamane, T. (1982) *Biophys. J. 38*, 217-226.
Stockton, G. W., & Smith, I. C. P. (1976) *Chem. Phys. Lipids 17*, 251-263.
Stockton, G. W., Polnaszek, C. F., Leitch, L. C., Tulloch, A. P., & Smith, I. C. P. (1974) *Biochem. Biophys. Res. Commun. 60*, 844-850.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954-966.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711-733.
Taylor, M. G., & Smith, I. C. P. (1980) *Biochim. Biophys. Acta 599*, 140-149.
Tilcock, C. P. S., Bally, M. B., Farren, S. B., & Cullis, P. R. (1982) *Biochemistry 21*, 4596-4601.
van Ginkel, G., Korstanje, L. J., van Langen, H., & Levine, Y. K. (1986) *Faraday Discuss. Chem. Soc. 81*, 49-61.
Vincent, M., & Galley, J. (1984) *Biochemistry 23*, 6514-6522.
Vist, M. R. (1984) Masters Thesis, University of Guelph, Guelph, Ontario, Canada.
Westerman, P. W., Vaz, M. J., Strenk, L. M., & Doane, J. W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2890-2894.
Wittebort, R. J., Olejniczak, E. T., & Griffin, R. G. (1987) *J. Chem. Phys. 86*, 5411-5420.
Wittebort, R. J., Blume, A., Huang, T.-H., Das Gupta, S. K., & Griffin, R. G. (1982) *Biochemistry 21*, 3487-3502.
Wolber, P. K., & Hudson, B. S. (1981) *Biochemistry 20*, 2800-2810.
Worchester, D. L., & Franks, N. P. (1976) *J. Mol. Biol. 100*, 359-378.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 276-287.

Thermodynamics of Thermal and Athermal Denaturation of γ-Crystallins: Changes in Conformational Stability upon Glutathione Reaction†

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ABSTRACT: The conformational stabilities of bovine lens γ-crystallin fractions II, IIIA, IIIB, and IVA and those modified with glutathione were compared by studying the thermal and guanidine hydrochloride (Gdn-HCl) denaturation behavior. The conformational state was monitored by both far-UV CD and fluorescence measurements. All the γ-crystallins studied showed a sigmoidal order–disorder transition with varied melting temperatures. The thermal denaturation of these proteins is reversible up to a temperature 3 or 4 °C above $T_{1/2}$; above this temperature, irreversible aggregation occurs. The validity of a two-state approximation of both thermal and Gdn-HCl denaturation was tested for all four crystallins, and the presence of one or more intermediates was evident in the unfolding of IVA. $\Delta G_D^{H_2O}$ values of these crystallins range from 4 to 9 kcal/mol. Upon glutathione treatment IVA showed the maximum decrease in $T_{1/2}$ by ~9 °C and in $\Delta G_D^{H_2O}$ value by 29%; the smallest decrease in $T_{1/2}$ was for IIIA by 2 °C and in $\Delta G_D^{H_2O}$ by 15%. We have demonstrated that the glutathione reaction can dramatically reduce the conformational stability of γ-crystallins and, thus, that the thermodynamic quantities of the unreacted crystallins can be used to evaluate the stability of these proteins when modified during cataract formation.

The soluble protein components of the mammalian lens fiber cells are designated as α-, β-, and γ-crystallins. The γ-crystallins are low molecular weight (M_r 20000–21000), monomeric proteins consisting of several gene products. The major crystallins of the bovine lens are γ-II, γ-IIIA, γ-IIIB, and γ-IVA with more than 75% sequence homology (Croft, 1973; Harding & Dilley, 1976; Bloemendal, 1982; Schoenmakers et al., 1984). X-ray studies have suggested that these proteins have a remarkably symmetrical structure that confers stability (Blundell et al., 1981, 1984; Chirgadze et al., 1981; Wistow et al., 1983). We have shown, however, that the microenvironments of tryptophan, tyrosine, and cysteine residues of γ-crystallins in solution vary greatly (Mandal et al., 1985, 1987a; Mandal & Chakrabarti, 1988). γ-Crystallins differ from their α and β counterparts in cryoprecipitation (Siezen et al., 1985a) and photoinduced (Bose et al., 1985, 1986; Chakrabarti et al., 1986; Mandal et al., 1986, 1988; Chakrabarti & Mandal, 1987; Kono et al., 1988) changes, and the various γ fractions differ in susceptibility to cryoprecipitation and ease and extent of photoaggregation (Kono et al., 1988; Mandal et al., 1988).

The stability of lens proteins is of particular interest because, unlike most other proteins, they cease turning over shortly after

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